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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 39/08, 39/116, 9/00

A1

(11) International Publication Number: WO 93/21950

(43) International Publication Date: 11 November 1993 (11.11.93)

(21) International Application Number: PCT/GB93/00880

(22) International Filing Date: 28 April 1993 (28.04.93)

(30) Priority data:
9209118.0 28 April 1992 (28.04.92) GB

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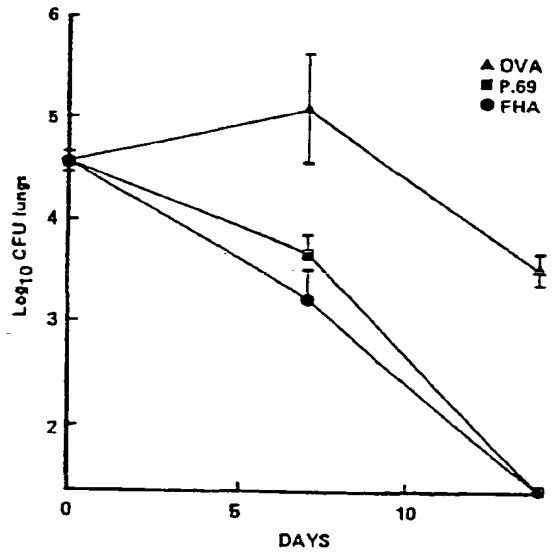
(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

73

(54) Title: VACCINE COMPOSITIONS FOR MUCOSAL DELIVERY



(57) Abstract

The invention provides the use of an antigen which is a mucosally immunogenically active substance comprising the 50kD C fragment of tetanus toxin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion for the manufacture of a vaccine composition for administration to a mucosal surface to induce an immune response in the mucosal surface against tetanus infection. The vaccine composition preferably contains the P.69 outer membrane protein of *B.pertussis*, and *B.pertussis* filamentous haemaglutinin. The invention also provides vaccine compositions *per se* and a method of treating tetanus and optionally whooping cough using the vaccine compositions.

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Vaccine compositions for mucosal delivery

The present invention relates to vaccine compositions for delivery to mucosal surfaces, and to a method of inducing, in a mammal, an immune response to an antigen or a mixture of antigens by delivering the antigen or mixture of antigens to a mucosal surface of the mammal.

More particularly, the present invention relates to vaccine compositions for inoculating a mammal such as a human against tetanus and B.pertussis infections.

It has long been the practice of clinicians to immunise human infants against a variety of common diseases by means of mixed vaccines which are directed against a plurality of diseases. For example, multiple-component vaccine compositions directed against diphtheria, tetanus and whooping cough have been available for a considerable number of years. Such vaccines have hitherto been administered by injection. The advantages of multiple-component vaccines are readily apparent in

that the patient (usually an infant) is subjected to a much smaller number of potentially distressing injections than would otherwise be the case.

The majority of infectious diseases are initiated by contact with a mucosal surface. The infecting agent may remain at or within the mucous membranes during the course of the infection or may penetrate into the body and localise at other sites. The importance of the mucous membranes in the first line of defence against infectious disease can be gleaned from the fact that 90% of the lymphocytes of the body underlie such surfaces. Priming mucosal surfaces by immunisation so that they respond vigorously and effectively control pathogenic organisms they encounter would be advantageous. Unfortunately traditional immunisation regimes are ineffective at eliciting mucosal responses. The systemic and local (mucosal) immune systems appear to be compartmentalised and in general do not impinge on one another; that is parenteral immunisation with non-living vaccines stimulates mucosal immune responses weakly if at all. Mucosal immunisation (oral or intranasal) can evoke serum antibodies but this is usually less effective than parenteral immunisation. The immunocytes of the different mucous membranes form a vast intercommunicating network, termed the common mucosal immune system, such that topical immunisation of one surface (e.g. the gastrointestinal tract) may lead to an immune response at that surface and also distance surfaces such as the respiratory tract.

Manclark and Shahin (US Patent application number 07/532 327, filed 5.6.90 - available through the US Department of Commerce, National Technical Information Service, Springfield, VA 22161, USA) - have described the intranasal and intraduodenal administration of filamentous hemagglutinin (FHA) obtained from Bordetella pertussis and have illustrated that FHA is an effective mucosal immunogen. Manclark and Shahin speculated in USSN 07/532,327 that the 69-kD outer membrane protein (P69) of B.pertussis would also be an effective mucosal immunogen, but presented no experimental data to show that this was the case.

The fact that there are very few mucosal vaccines commercially available indicates that there are problems with developing such vaccines Many non-living soluble antigens, particularly those used traditionally by immunologists, such as ovalbumin (OVA) and Keyhole Limpet Haemocyanin (KLH), are poor mucosal immunogens. Large doses of such antigens are necessary to induce any responses but large doses can also cause tolerance in the individual to subsequent parenteral exposure to antigen, a condition known as oral tolerance. Some microbial components such as the cholera toxin (CT) or E.coli heat-labile toxin (LT) or the non-toxic binding portions of these toxins (CT-B and LT-B) have been found to be potent mucosal immunogens eliciting strong secretary and circulating antibodies, but the reason why such molecules are good mucosal immunogens has not yet been fully

elucidated. One property that may be important is the ability of these molecules to bind to mucosal epithelial cells via certain surface receptors, although it has been found in studies by others that there is not necessarily a correlation between the ability of an antigen to bind to eucaryotic cells and its mucosal immunogenicity.

Thus, as far as we are aware, there is currently no way of predicting with any certainty whether a given antigen will possess good mucosal immunogenicity.

We have now found that certain molecules make excellent mucosal immunogens and such components can be utilised in the development of a mucosally (intranasally or orally) delivered vaccine against the diseases whooping cough and tetanus. In particular, we have found that the P69 outer membrane protein (P69 - also known as pertactin) from B.pertussis and the non-toxic immunogenic 50Kd portion of tetanus toxin (C-Fragment) from C.tetanii are highly immunogenic when given intranasally. C-Fragment and P.69 were generated by DNA recombinant technology. Recombinant C-Fragment and P.69 produced from E.coli and yeast have been demonstrated to be immunogenic and protective in mice, see M.Roberts et al, Recombinant P.69/pertactin: immunogenicity and protection of mice against Bordetella pertussis infection; Vaccine 10, 43 (1992); and see also N.F. Fairweather et al, Infection and Immunity, 55, 2541 (1987).

In a first aspect, the invention provides the use of a mucosally immunogenically active substance comprising the 50kD C fragment of tetanus toxin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion, for the manufacture of a vaccine composition for immunising a patient against tetanus infection.

In one particular embodiment of the invention, there is provided the use of a mixture of antigens for the manufacture of a vaccine composition for administration to a mucosal surface to induce an immune response in the mucosal surface against each of the said antigens, the mixture of antigens comprising:

(a) a mucosally immunogenically active substance comprising the 50kD C fragment of tetanus toxin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion; and

(b) a mucosally immunogenically active substance comprising the P.69 outer membrane protein of B.pertussis; an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

In a preferred embodiment the invention provides the use of a mixture of antigens as hereinbefore defined but wherein said mixture comprises in addition to (a) and (b);

(c) a mucosally immunogenically active substance comprising B.pertussis filamentous haemaglutinin, an

immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

In a further aspect, the invention provides a vaccine composition for application to a mucosal surface, the composition comprising antigen (a) or a mixture of antigens as hereinbefore defined and a pharmaceutically acceptable carrier.

In another aspect the invention, provides a method of immunising a host such as a mammal, (e.g. human) against infection, which method comprises administering an effective amount of antigen (a), or a mixture of antigens as hereinbefore defined, directly to a mucosal surface in the host to induce in said mucosal surface an immune response to each said antigen.

The mucosal delivery compositions of the present invention can be formulated, for example, for delivery to one or more of the oral, gastro-intestinal, and respiratory (e.g. nasal and bronchial) mucosa.

Where the composition is intended for delivery to the respiratory (e.g. nasal or bronchial) mucosa, typically it is formulated as an aqueous solution for administration as an aerosol or nasal drops, or as a dry powder, e.g. for inhalation.

Compositions for administration as nasal drops may contain one or more excipients of the type usually

included in such compositions, for example preservatives, viscosity adjusting agents, tonicity adjusting agents, buffering agents and the like.

The antigenic preparations of the present invention may also take the form of compositions intended to deliver the mixture of antigen to mucosal surfaces in the gastrointestinal tract. It is preferred that such compositions are provided with means for preventing degradation of the antigens by the gastric juices. For example, the compositions may take the form of capsules, e.g. microcapsules, in which the antigens are retained within a protective matrix or coating formed from an appropriate protective polymer such as a poly (glycolide), poly (lactide-co-glycolide), polyacryl starch, or pH-dependent coatings such as the polyacrylates or hydroxypropylmethyl cellulose phthalate.

The antigens may take the form of the tetanus toxin C fragment per se, the P.69 protein per se, or the B.pertussis haemaglutinin per se. Or it may take the form of a larger molecule containing one or more of the aforesaid antigens, immunogenically active fragments thereof, or derivatives formed by amino acid deletion, substitution and insertion, provided that the larger molecule is immunogenically active when administered directly to the mucosa.

The antigen or mixture of antigens typically is selected such that it is non-toxic to a recipient thereof at concentrations employed to elicit an immune response.

In one embodiment, two more of the antigens forming the mixture may be presented in a single molecule. Such a molecule may be prepared by recombinant methods by preparing a DNA construct containing genes coding for two or more of the antigens and expressing in a suitable host in accordance with known methods.

The individual antigenic substances making up the compositions of the invention may each also act as carriers for one or more other antigens. For example, an antigen such as the P.69 outer membrane protein or C-Fragment may be coupled to another antigen, and examples of such "other" antigens include Haemophilus group B and meningococcal polysaccharide antigens.

In order to enhance the mucosal immunogenicity of the mixture of antigens or any component antigen thereof or appropriate immunogenic fragments thereof, they may be incorporated into appropriate carriers, for example virosomes, or the antigens or immunogenic fragments thereof may be expressed in suitable attenuated carrier strains of Salmonella. Immunogenicity may also be enhanced by incorporating appropriate mucosal adjuvants such as cholera toxin or E.coli heat-labile toxin, genetically detoxified variants thereof or their binding (B) sub-units in the vaccine.

The vaccine composition may optionally contain another mucosally immunogenically active portion of the tetanus toxin molecule. In addition, the mixed vaccine may contain one or more further mucosally immunogenically

active antigens.

In one embodiment, the vaccine composition may, in addition to non-toxic immunogenic forms of tetanus toxin and pertussis antigens, contain non-toxic immunogenic forms of diphtheria toxin and immunogenic forms of Haemophilus influenzae group B polysaccharide (HiB), thereby providing a mucosal diphtheria-tetanus-pertussis (DTP) vaccine or DTPHiB vaccine.

The P.69 outer membrane protein of B.pertussis is a protein of approximately 61 KD molecular weight; see A.J.Makoff et al, "Protective surface antigen P.69 of Bordetella pertussis: its characteristics and very high level expression in Escherichia coli", Bio-Technology, 8, 1030 (1990).

It can be prepared and isolated according to the method disclosed in P.Novotny et al: The Journal of Infectious Diseases, 164, 114 (1991), or recombinant material prepared from E.coli by the method given in the article by A.J.Makoff et al referred to above. It can bind to eukaryotic cells.

Purified B.pertussis filamentous haemaglutinin usually contains polypeptides of differing molecular weight ranging from 98-220 KD, and can be isolated and purified from cell culture supernatants of B.pertussis, for example as described in the article by P. Novotny et al referred to above. The filamentous haemaglutinin is able to bind to eukaryotic cells and cause haemaglutination of sheep erythrocytes.

The C fragment of tetanus toxin is a peptide of approximately 50KD molecular weight which can be isolated and purified from E.coli by the method described in A.J.Makoff et al, Bio/Technology, 7, 1043 (1989). The C fragment is characterised by an ability to bind the eukaryotic cells possessing the trisialoganlioside G_T1b and by an ability to elicit protection in mice against lethal challenge with tetanus toxin.

The antigenic molecules of the present invention can be prepared by isolation and purification from the organisms in which they occur naturally, or they may be prepared by recombinant techniques and expressed in a suitable host such as E.coli in known manner. When prepared by a recombinant method or by synthesis, one or more insertions, deletions, inversions or substitutions of the amino acids constituting the peptide may be made.

Each of the aforementioned antigens is preferably used in the substantially pure state. The quantity of the mixture of antigens administered will depend, in part, upon the purity of the individual antigens. Thus, for a substantially pure form of the P.69 outer membrane protein, a dose in the range from about 1-100 microgrammes/dose typically would be administered to a human, the actual amount depending on the immunogenicity of the preparation in humans when applied to mucosal surfaces.

For a substantially pure form of the B.pertussis filamentous haemaglutinin, and the 50KD C fragment of

tetanus toxin, a typical dose range would be of the order given above in respect of P.69 protein. In a typical immunisation regime employing the antigenic preparations of the present invention, the vaccine may be administered in several doses (e.g. 1-4), each dose containing 1-100 microgrammes of each antigen. The immunisation regime may involve immunisation purely by the mucosal route or by a combination of mucosal and parenteral immunisation. The dosage will in general depend upon the immunogenicity of the different antigens when applied to the respiratory or gastrointestinal tracts of humans.

The invention will now be illustrated in greater detail by reference to the specific embodiments described in the following examples.

The examples are intended to be purely illustrative of the invention and are not intended to limit its scope in any way.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the growth of B.pertussis in the lungs of intranasally immunised mice. Mice were immunised intranasally with two doses of antigen on day 0 and day 29 and then aerosol challenged with B.pertussis 14 days later (day 43). The counts represent the means of 4 pairs of lungs ± SEM.

Figure 2 illustrates the local immune response in the lungs of mice in response to intranasal immunisation and challenge. Lymphocytes were purified from the lungs of

groups of mice after intranasal immunisation and aerosol challenge and the number of cells secreting antibody to the immunising antigen was determined by the ELISPOT assay. Counts represent the response of lymphocytes pooled from 4 mice.

Figure 3 illustrates the primary antibody response to pertussis antigens in the lungs of mice following aerosol challenge. Lymphocytes from Lungs removed 10 days after aerosol challenge were assayed for antibody production against P69 or FHA. Mice immunised with one antigen were tested against the other antigen. Counts represent the response of lymphocytes pooled from groups of 4 mice.

EXAMPLE 1 PERTUSSIS COMPONENTS

Mice were immunised with 12μg of either P.69, FHA or OVA on day 1 and day 29. Mice were then aerosol challenged with Bordetella pertussis on day 43 (14d post boost). Lungs were removed from groups of mice at periods after challenge, homogenised and viable bacterial counts performed to determine the growth of B.pertussis in the respiratory tract. The results are shown in Figure 1. Bacteria increased in numbers in the lungs of control mice (OVA) during the first seven days. In contrast, in both P.69 and FHA immunised animals bacterial number declined during this period and at the day 7 time point the levels of bacteria in the lungs of immunised mice were only 1-4% of those in the control group. By day 14 bacteria had almost cleared from the lungs of the immunised mice but were still present in large numbers in the OVA immunised

mice.

The local immune response in the lungs of mice was analysed by enumerating cells secreting antibody against FHA, P.69 or OVA amongst lymphocytes isolated from the lungs of mice by "ELISPOT". There were low numbers of antibody secreting cells (ASC) producing antibody against the immunising antigens after the second (Figure 2). Following aerosol challenge of ASC in the lungs of mice immunised with FHA or P.69, but not OVA, increased by several orders of magnitude (Figure 2). Cells secreting antibody of IgG, IgA and IgM isotypes were detected.

The increase in ASC following aerosol challenge may represent a primary response to the antigens present in the challenge organisms or a boost of the response in immunised mice. To see which of these options was correct, lung lymphocytes were screened against antigens which the mice had not encountered until challenge (FHA immunised mice were tested against P.69 etc). As can be seen from Figure 3, the responses were similar in all mice and comparisons with the same time point (d23) in Figure 1 shows that prior intranasal immunisations boost the response several fold.

The serum response was studied using ELISA and the results are shown in Table 1 below:

Table 1. Serum anti-pertussis antibody response of intranasally immunised mice

ANTIBODY TITRE[a]

Days post-immunisation

	8		20		24	
Group	FHA	P.69	FHA	P.69	FHA	P.69
FHA	<50	<50	400	100	1600	100
P.69	<50	<50	<50	200	200	400
OVA	<50	<50	<50	<50	100	200

[a] Determined by Elisa

[b] Mice aerosol challenged d 14

There were no detectable antibodies against FHA of P.69 prior to aerosol challenge (d8). There were no anti-OVA antibodies to serum of OVA immunised mice (data not shown). Following challenge (>d14) the anti-FHA response did exhibit large increase in FHA immunised mice. This increase was specific, the anti-P.69 response did not increase proportionally in these mice. There was a small increase in anti-P.69 titre in P.69 immunised mice compared to FHA or P.69 immunised mice. This demonstrated that previous respiratory exposure to FHA and, possibly, P.69 can prime the mice to mount an amnestic response serum response upon contact with the pathogen.

EXAMPLE 2 TETANUS COMPONENT

Groups of 10 mice were immunised intranasally with one or two doses of 20µg of C fragment or OVA as a control. 28 days after the final immunisation mice were challenged with 500LD_{50} of tetanus toxin and their survival monitored for 4 days, the results are shown in Table 2 below. Survival was enhanced in mice receiving 1 or 2 doses of C fragment compared to the control mice. All of the control mice were dead the day after challenge. Protection was greatly enhanced by giving 2 doses of C fragment, by day 4 post-challenge 80% of the mice in the 2 dose group were still alive compared to 10% in the mice receiving a single dose.

Table 2. Protection of mice against tetanus toxin challenge by I/N immunisation with C fragment

Group	Dose	No of Mice	Survival post-challenge			
			Day			
			2	3	4	5
1. C-frag	20µg	10	3	3	2	1
2. C-frag	2 x 20µg	10	10	8	8	8
3. Ova	2 x 20µg	10	0	0	0	0

SERUM RESPONSE

The mice receiving 2 doses of C fragment had a mean serum anti-C fragment titre of greater than 200 prior to challenge.

MATERIALS AND METHODS

ANTIGENS

P. 69 was synthesised intracellularly in E.coli and purified as described in A.J.Makoff et al, Bio/Technology 8, 1030 (1990). FHA was provided by SKB under and exchange of reagents agreement. C fragment was produced from E.coli as in A.J.Makoff et al, Bio/Technology 7, 1043 (1989). Antigens were diluted in PBS immediately prior to immunisation.

IMMUNISATION

Adult (6-8 weeks) BALB/c mice were anaesthetised with metathane. 30µl of antigen solution was added to the external nares of mice (15µl/nares) as they recovered consciousness. Antigen was taken into respiratory tract by inhalation.

AEROSOL CHALLENGE WITH B.PERTUSSIS

Mice were placed in cages on a rotating carousel in a plastic exposure chamber as described in P.Novotny et al. Development for Biological Standards, 61, 27 91985). A bacterial suspension in PBS was prepared from 2-to 3-day old cultures of B.pertussis BBC26 grown on CW blood agar plates. The mice were exposed to an aerosol (generated from the bacterial suspension) of 2 x 10^9 Colony-forming units (CFU) in PBS by a Turret mouthpiece tubing operated by a System 22 CR60 high-glow compressor (Medic-Aid),

Pagham, West Sussex, UK) giving a very fine mist at a dynamic flow of 8.5 litres/min. The generated mist was drawn through a chamber by a vacuum pump at a passage of ca.12L of air per mist mixture per min, which maintained 70% relative humidity in the chamber. The exposure to aerosol lasted 30 min; a period of 10 min then allowed the chamber to clear.

The course of the infection was assessed by performing counts of viable bacteria in lungs. Groups of four mice were removed at intervals and killed by cervical dislocation, and their lungs were aseptically removed and homogenised in a Potter-Elvehjem homogenizer with 2ml of PBS. Dilutions of the homogenate were spotted onto Cohen-Wheeler (CW) blood agar plates and the number of CFU was determined for each set of lungs.

TETANUS CHALLENGE

Mice were challenged with 500 LD_{50}'s of tetanus toxin subcutaneously and observed regularly for 5 days.

ELISA

Serum anti-P.69, anti-FHA and anti-C fragment antibodies were measured using an enzyme-linked immunosorbent assay (ELISA). Antigen (50µl; 1 g/ml in phosphate-buffered saline, pH7.2) was absorbed onto 96-well microtitre plates (EIA 'Costar' NBL, Northumbria, UK) by incubation at 4°C overnight. Wells were aspirated and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T; Sigma), and then blocked with 3% (w/v) bovine serum albumin (BSA; Sigma) in PBS. After washing, 50µl of

test serum appropriately diluted in PBS-T-0.1% BSA was added per well. After incubation at 37°C for 2h, the wells were washed and incubated at room temperature with 50µl of substrate (0.04% O-phenylenediamine hydrochloride; Sigma) dissolved in phosphate citrate buffer (pH5.0[24mM citrate, 64mM disodium hydrogen phosphate] containing 40µl hydrogen peroxide). The reaction was terminated by the addition of 50µl 1M sulphuric acid. Plates were read in a Titertek Multiscan MCC ELISA reader at 492nm.

The titre was expressed as the reciprocal of the highest dilution of test serum that gave an absorbance reading twice that of the similarly diluted pre-bleed serum. Absorbance values below 0.1 were discarded.

ELSIPOT Assay for specific antibody secreting cells (ASC) in murine lungs.

Local antibody production in the murine lung was determined using the ELISPOT technique. Lymphocytes were isolated from murine lungs as follows: Lungs were washed briefly in PBS to remove traces of blood and then were finely chopped with a scalpel blade. 1ml of PBS containing 10mM $MgCl_2$, 0.5U/ml collagenase A (Boehringer Mannheim, Lewes, UK) and 0.25mg/ml DNase 1 (Boehringer) was added for each pair of lungs and incubated at 37°C with gentle agitation for 45 min. The mixture was then passed through a 40 gauge mesh. Lumps were pressed through the mesh with the plunger from a 5ml syringe. The cell suspension was placed in a centrifuge tube and allowed to stand for several minutes to allow large debris to settle. The

supernatant was removed and the cells were pelleted and washed several times. Red cells and non-viable cells were removed by centrifugation on a Ficol-Isopaque gradient (LSM,Flow Laboratories Ltd, Herts, UK). After washing cell viability was determined by Trypan Blue exclusion. Cells were finally suspended in RPM11640 complete Medium (10% foetal calf serum, penicillin 100IU/ml, streptomycin 100 g/ml, L-glutamine 2mm; Flow).

The ELSIPOT assay was performed as follows. Briefly, 24-well tissue culture plates (Costar) were coated overnight with P.69, FHA or OVA (0.5ml of 1 g.ml in PBA) after washing and blocking 0.5ml volumes of dilutions of the lymphocyte suspension in complete RPM_1 1640 were added to the wells and incubated at 37°C/10% CO_2 for 3h. After washing goat anti-mouse IgG, A or M (1/1000, Sigma) and rabbit anti-goat IgG-alkaline phosphatase (1/1000, Sigma) were added sequentially. Finally, substrate solution (0.5μ l of 1mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1,3-propanediol (AMP) buffer, Sigma) was added and plates were incubated until blue spots were visible under low power microscopy.

CLAIMS

1 The use of an antigen, (hereinafter referred to as "(a)"), which is a mucosally immunogenically active substance comprising the 50kD C fragment of tetanus toxin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion for the manufacture of a vaccine composition for administration to a mucosal surface to induce an immune response in the mucosal surface against tetanus infection.

2 The use according to Claim 1 wherein the vaccine composition further comprises:
(b) a mucosally immunogenically active substance comprising the P.69 outer membrane protein of B.pertussis; an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

3 The use according to Claim 1 or Claim 2 wherein the vaccine composition further comprises:
(c) a mucosally immunogenically active substance comprising B.pertussis filamentous haemaglutinin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

4. The use according to any one of Claims 1 to 3 wherein

the vaccine composition is for administration to the respiratory (e.g. nasal or bronchial), or gastrointestinal mucosa.

5. A vaccine composition in a form suitable for application to a mucosal surface, the composition comprising the mucosally immunogenically active substance (a) as defined in Claim 1, and optionally the substance (b) and/or the substance (c) as defined in Claim 2 and Claim 3; and
(d) a pharmaceutically acceptable carrier therefor.

6. A vaccine composition according to Claim 5 which is adapted for delivery to the respiratory (e.g. nasal or bronchial) mucosa or gastrointestinal mucosa.

7. A vaccine composition according to Claim 6 in the form of a solution for administration as a nasal spray or nasal drops.

8. A vaccine composition according to Claim 7 in the form of nasal drops, said composition comprising means for administering drops to the nasal mucosa.

9. A vaccine composition according to Claim 6 wherein the antigens are encased in a protective coating to prevent degradation by the gastric juices.

10. A vaccine composition accordind to Claim 9 which is in the form of microspheres or a tablet or capsule coated with, or comprising a matrix formed from, a protective polymeric substance.

11. A method of immunising a host such as a mammal against tetanus and B.pertussis infection, which method comprises administering directly to a mucosal surface of said host, in an amount effective to induce an immune response in said surface, a vaccine composition comprising:
(a) a mucosally immunogenically active substance comprising the 50kD C fragment of tetanus toxin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion; and
(d) a pharmaceutically acceptable carrier.

12. A method according to Claim 11 wherein the vaccine composition further comprises:
(b) an antigen which is a mucosally immunogenically active substance comprising the P.69 outer membrane protein of B.pertussis; an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

13. A method according to Claim 11 or Claim 12 wherein the vaccine composition further comprises:
(c) a mucosally immunogenically active substance comprising

B.pertussis filamentous haemaglutinin, an immunogenic fragment thereof, or a derivative thereof formed by amino acid deletion, substitution or insertion.

14. A method according to any one of Claims 11 to 13 wherein the antigen or mixture of antigens is administered directly to the respiratory (e.g. nasal or bronchial) mucosa or gastrointestinal mucosa.

15. A method according to Claim 14 wherein the antigen or mixture of antigens is administered in the form of nasal drops, or a nasal spray or dry powder for inhalation.

16. A method according to Claim 14 wherein the antigen or mixture of antigens is administered orally as a composition in which each antigen is provided with a protective coating to prevent degradation by the gastric juices.

17. The use according to any one of Claims 1 to 4 wherein the vaccine composition contains, in addition to the antigens (a), (b) and (c) as appropriate, optionally a non-toxic immunogenic form of tetanus toxin, and/or a further antigen or antigens selected from a non-toxic mucosally immunogenic form of diphtheria toxin and Haemophilus influenzae group B polysaccharide.

18. A vaccine composition according to any one of Claims 5 to 10 which contains, in addition to the antigens (a), (b) and (c) as appropriate, optionally a non-toxic immunogenic form of tetanus toxin, and/or a further antigen or antigens selected from a non-toxic mucosally immunogenic form of diphtheria toxin and Haemophilus influenzae group B polysaccharide.

19. A method according to any one of Claims 11 to 16 wherein there is administered to the mucosal surface a vaccine composition which contains, in addition to the antigens (a), (b) and (c) as appropriate, optionally a non-toxic immunogenic form of tetanus toxin, and/or a further antigen or antigens selected from a non-toxic mucosally immunogenic form of diphtheriatoxin and Haemophilus influenzae group B polysaccharide.

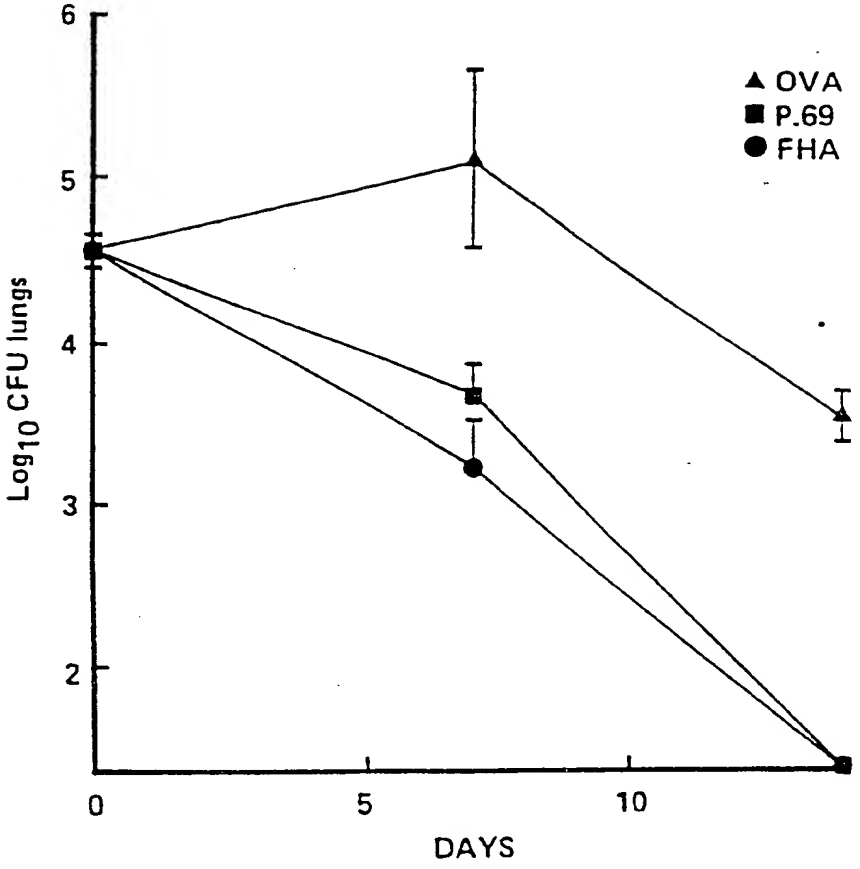


FIG. 1

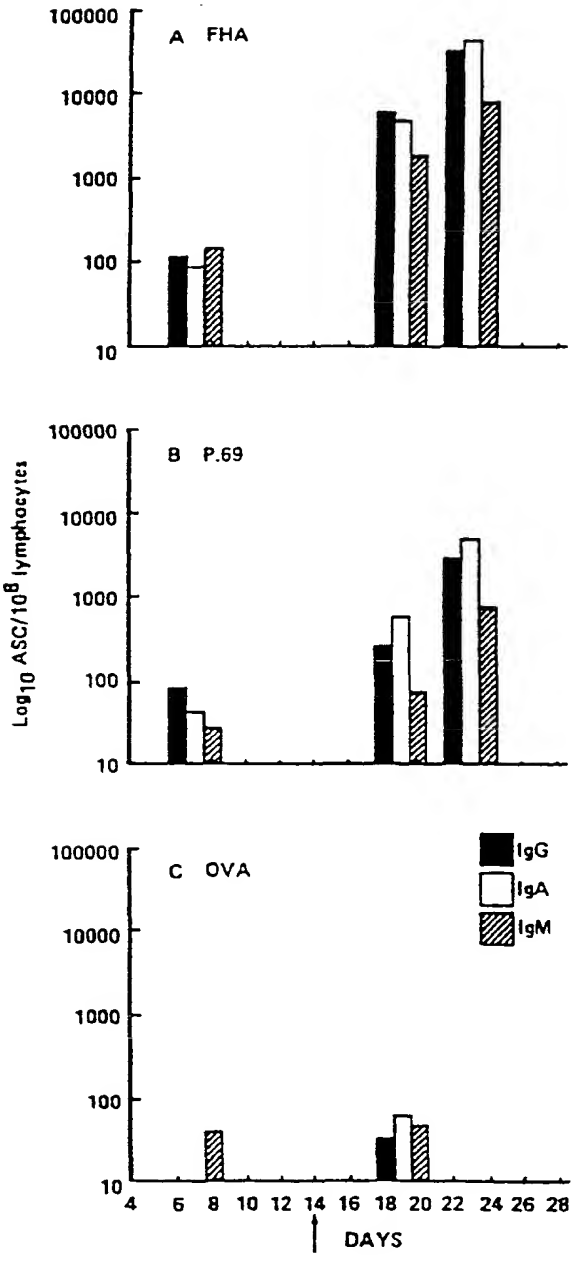
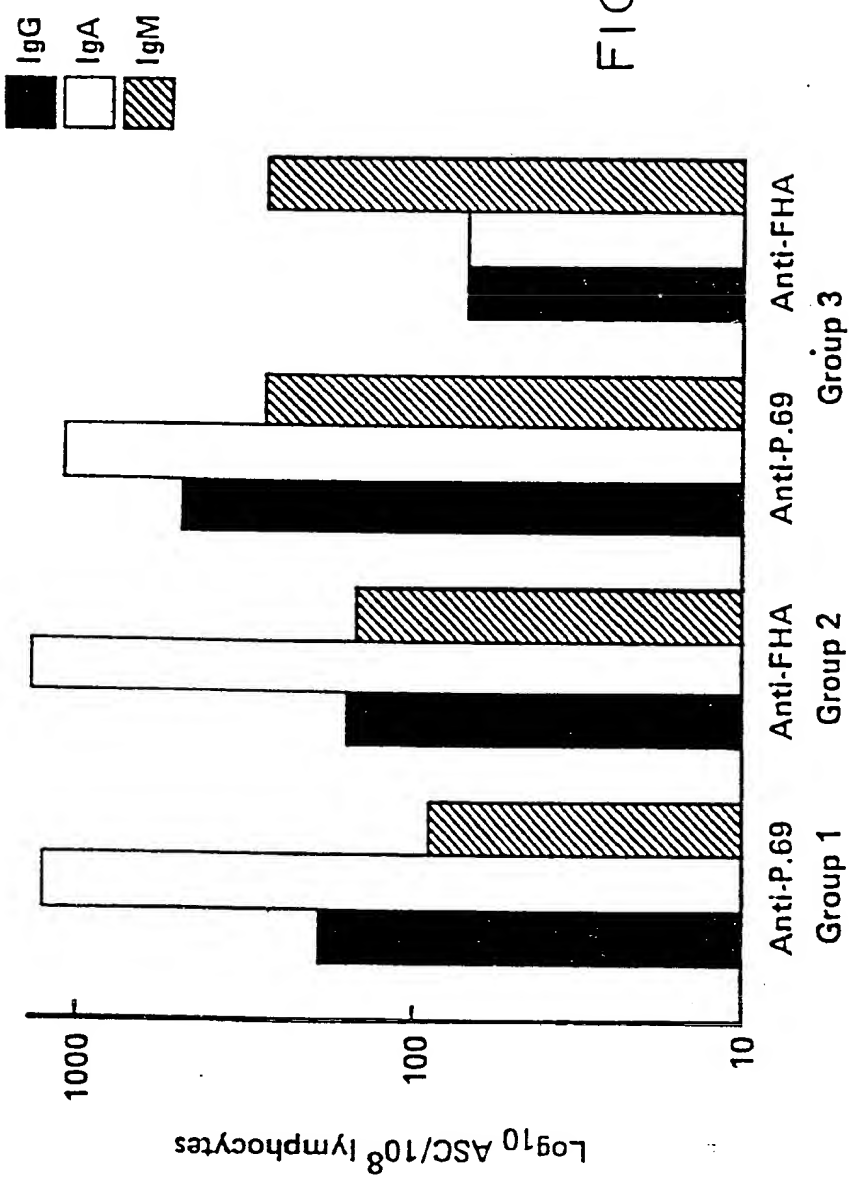

A FHA
B P.69
C OVA
IgG
IgA
IgM
100000
10000
1000
100
10
Log_{10} ASC/10^8 lymphocytes
4 6 8 10 12 14 16 18 20 22 24 26 28
DAYS

FIG. 2

FIG. 3


IgG
IgA
IgM
Anti-P.69
Group 1
Anti-FHA
Group 2
Anti-P.69
Group 3
Anti-FHA
1000
100
10
Log10 ASC/10^8 lymphocytes

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00880

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/08; A61K39/116; A61K9/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 209 281 (THE WELLCOME FOUNDATION LIMITED) 21 January 1987	1,4-6, 11,14, 17-19
Y	see page 7, paragraph 5 - page 8, paragraph 2 see page 11, paragraph 6 - page 12, paragraph 5 see page 22 - page 23; example 9	2,3, 7-10,12, 13,15,16
X	WO,A,9 015 871 (THE WELLCOME FOUNDATION LIMITED) 27 December 1990	1,4-6, 11,14
Y	see page 4, paragraph 2 - page 5, paragraph 3	2,3, 7-10,12, 13,15-19

-/--

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 JULY 1993	20 -08- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SITCH W.D.C.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	VACCINE vol. 10, no. 1, 1992, GUILDFORD,UK pages 53 - 60 CHATFIELD ET AL 'CONSTRUCTION OF A GENETICALLY DEFINED SALMONELLA TYPHI TY2 AROA,AROC MUTANT FOR THE ENGINEERING OF A CANDIDATE ORAL TYPHOID-TETANUS VACCINE'	1,4-6, 11,14
Y	see the whole document	2,3, 7-10,12, 13,15-19

X	INFECTION AND IMMUNITY vol. 58, no. 5, 1990, WASHINGTON D.C.,USA pages 1323 - 1326 FAIRWEATHER ET AL 'ORAL VACCINATION OF MICE AGAINST TETANUS BY USE OF A LIVE ATTENUATED SALMONELLA CARRIER'	1,4-6, 11,14
Y	see the whole document	2,3, 7-10,12, 13,15-19

Y	EP,A,0 462 534 (SCLAVO S.P.A.) 27 December 1991 see the whole document	2,3,12, 13,17-19

Y	WO,A,9 013 313 (THE WELLCOME FOUNDATION LIMITED) 15 November 1990 see whole document, and especially page 5,paragraph 5	2,3,12, 13

Y	DATABASE WPI Section Ch, Week 9135, Derwent Publications Ltd., London, GB; Class B04, AN 91-260180 & US,A,7 532 327 (NAT INST OF HEALTH) 23 July 1991 cited in the application see abstract	2,3,9, 10,12, 13,16

Y	DATABASE WPI Section Ch, Week 8807, Derwent Publications Ltd., London, GB; Class B04, AN 88-045809 & JP,A,63 002 932 (TEIJIN KK) 7 January 1988 see abstract	7,8,15

P,Y	EP,A,0 484 621 (AMERICAN CYANAMID COMPANY) 13 May 1992 see the whole document	2,3,12, 13,17-19

	-/--	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO,A,9 215 689 (THE WELLCOME FOUNDATION LIMITED) 17 September 1992 see page 9, line 27 - page 10, line 10; claims 1,9,10	1,4-11, 14-16
P,Y		2,3,12, 13,17-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB93/00880

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 11-16 and 19 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9300880
SA 73460

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/07/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0209281	21-01-87	AU-A- 5941486	08-01-87
		JP-A- 62051994	06-03-87
WO-A-9015871	27-12-90	EP-A- 0478602	08-04-92
		JP-T- 4506005	22-10-92
EP-A-0462534	27-12-91	CA-A- 2045071	22-12-91
		JP-A- 4368337	21-12-92
WO-A-9013313	15-11-90	EP-A- 0471726	26-02-92
		JP-T- 4505158	10-09-92
EP-A-0484621	13-05-92	None	
WO-A-9215689	17-09-92	AU-A- 1350892	06-10-92
		WO-A- 9215688	17-09-92